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DETECTION/QUANTIFICATION OF TARGETED NUCLEOTIDE CHAINS,  
AND DETECTION/QUANTIFICATION OF MULTI-FOLD NUCLEOTIDE  
CHAINS BY FLUORESCENCE

## 5      BACKGROUND OF THE INVENTION

## Field of the Invention

The present invention relates to  
detection/quantification of targeted nucleotide chains  
with specific base sequences therein and  
10      detection/quantification of multi-stranded nucleotide  
chains such as double-stranded nucleotide chains.

## 15      Related Background Art

In order to detect specific genes of organisms, in  
the detection of a double-stranded nucleotide hybrid of  
15      a targeted nucleotide chain and a nucleotide probe for  
the targeted nucleotide chain, a technology has been  
widely used of detecting/quantifying the double-  
stranded nucleotide chain in gel or solution using  
fluorescence dyes, such as ethidium bromide  
20      (hereinafter referred to as EB), which act on double-  
stranded nucleotide chains and increase their  
fluorescence intensity.

In recent years, while polymerase chain reaction,  
which replicates nucleotide chains with an enzyme, has  
25      been very commonly used to detect a very slight amount  
of nucleotide chains, a method has also been used which  
uses 2-methyl-4,6-bis(4-N,N-dimethylaminophenyl)

pyrylium salt, a dye emitting fluorescence only after acting on double-stranded nucleotide chains in solution, to detect the PCR amplification products (Nucleic Acid Research, 1995, Vol. 23, No. 8 1445-1446).

5 1446).

Further, quite recently, a fact has been known that there exist in organisms triple-stranded and quadruple-stranded nucleotide chains having special base sequences and those nucleotide chains play an important part in the replication of genes and the lifetime of cells in organisms.

These nucleotide chains form triple-stranded chains and quadruple-stranded chains and, in each chain, 3 or 4 bases in corresponding positions form a base trio or a base quartet in a plane.

Although there have been very few studies on dyes acting on these triple- and quadruple-stranded chains, in particular on fluorescence dyes, there is found in a collection of lecture resumes II (665) for the 72th 20 spring meeting of the Chemistry Society of Japan (1997) a description that 2-methyl-4,6-bis(4-N,N-dimethylaminophenyl) pyrylium salt acts on triple- and quadruple-stranded DNAs and emit fluorescence in a solution.

25 Further, besides of the above dye, there have been  
several dyes, including YOYO1 from Molecular Probe Co.,  
on the market in recent years.

Among these dyes, EB is generally considered to be an intercalator which enters the space between the two base pairs of double-stranded nucleotide chains, and there is found in a literature (Nucleic Acid Symposium Series No. 29 1993 83-84) a description that 2-methyl-4,6-bis(4-N,N-dimethylaminophenyl) pyrylium salt is also an intercalator. On the other hand, YOYO1 is generally considered to be a groove binding type fluorescence dye which enters the groove of double-stranded nucleotide chains.

The method, what is called solid-phase hybridization, is widely known which forms a hybrid on a solid phase by fixing a targeted nucleotide chain, which has a specific base sequence, for use in detection of a specific gene or fixing a probe nucleotide chain having a base sequence complementary to a specific site of the base sequence of the targeted nucleotide chain on a solid phase and by reacting the same with the corresponding probe nucleotide chain or targeted nucleotide chain to detect the hybrid thereof. And there have been developed various detecting techniques.

The most typical technique is the Southern hybridization technique, which forms a hybrid on, for example, a nylon filter by transferring a nucleotide chain, which is developed according to their length utilizing gel electrophoresis, to the nylon filter,

denaturing the same to be single-stranded, and allowing a probe nucleotide chain labeled with a radioisotope to act on the same, so as to detect the formed hybrid thereof by using autoradiography.

5       And in order to overcome the problems attendant to the use of radioisotope, an improved technique has been put to practical use which uses chemiluminescence as a detecting means.

10       In recent years, as means for detecting a specific gene more simply and easily, a technique has also been developed which forms a hybrid on a micro-plate by fixing a probe nucleotide chain on the micro-plate and allowing a mRNA targeted nucleotide chain to act on the same, so as to detect the formed hybrid thereof with a 15       dye, for example, ethidium bromide (hereinafter referred to as EB), which acts specifically on double-stranded nucleotide chains and thereby increase its fluorescence intensity (published Japanese Laid-Open Application of PCT Patent Application No. 7-506482).

20       As described above, there have been known dyes which act on double-stranded nucleotide chains or multi-stranded nucleotide chains in solution and thereby increase their fluorescence intensity or emit fluorescence for the first time; however, what is known 25       about them is just the behavior in solution.

Usually for the determination of the fluorescence characteristics (intensity, excitation/fluorescence

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spectrum, etc.) of fluorescence dyes, measurements are made for about 1 to 4 mL of sample solution with a dye dissolved therein placed in measuring glass cells, generally with a spectrofluorometer. Micro-cells of about 200  $\mu$ L are also commercially available. And, lately, devices are also commercially available from several manufacturers which make measurements automatically and continuously for sample solutions placed in about 100 to 250  $\mu$ L wells up to 96 of a plastic micro-plate (for example, Cyto Flour, by Nippon Perceptive Ltd.).

In any of these measurements, since measurements are made for the sample solution in solution as described above, the containers for use in the measurements are restricted, further, when making measurements using a micro-plate, if the sample is illuminated with excitation light from the solution surface side, there occurs a problem of causing diffused reflection and scattering on the solution surface, and the illumination has to be conducted from the plate back side.

Further, when the amount of the sample solution is as small as 0.5 to 5  $\mu$ L, the containers for use in the measurements are limited. In addition, when trying to measure fluorescence with a microscope, there arise not only a problem of excitation direction as described above, but also a problem of permitting the sample to

be dried and the fluorescence of the same to be quenched, even in a typical fluorescence dye, FITC (fluorescein isothiocyanate).

## 5 SUMMARY OF THE INVENTION

In light of the above problems, the present inventors concentrated their energies on an intensive investigation of the detection/quantification of targeted nucleotide chains, in particular, of multi-stranded nucleotide chains including double-stranded nucleotide chains, and have finally made the present invention.

According to one aspect of the present invention,  
there is provided a method for dry  
detection/quantification of targeted nucleotide chains  
comprising the steps of:

(1) realizing a state in which a hybrid (C) of a certain amount of targeted nucleotide chain (A), which is derived from a sample solution and subjected to detection or quantification, and a probe nucleotide chain (B), which has a base sequence complementary to a specific site of the base sequences of the targeted nucleotide chain, is formed on a solid-phase substrate by mutually reacting the above two types of nucleotide chains with each other, and in which there exists a fluorescence dye (D), which acts on the hybrid (C), thereby emits fluorescence or increases its

fluorescence intensity, and is capable of continuing to emit fluorescence even in the dried state while acting on the hybrid;

5 (2) drying the hybrid (C) and the fluorescence dye (D) on the substrate; and

(3) measuring the fluorescence emitted from the fluorescence dye (D), as a measuring means, after the drying operation.

10 According to another aspect of the present invention, there is provided a method for detection/quantification of multi-stranded nucleotide chains comprising the steps of:

15 (1) adding to a sample solution, which is subjected to detection/quantification of a multi-stranded nucleotide chain, a fluorescence dye having a fluorescence characteristic of emitting fluorescence or increasing its fluorescence intensity in the presence of a multi-stranded nucleotide chain and capable of maintaining the fluorescence characteristic even in the 20 dried state;

(2) placing a known amount of the sample solution with the fluorescence dye added thereto on a clean observation substrate so as to dry the same; and

25 (3) measuring the fluorescence emitted from the dried sample and detecting/quantifying the multi-stranded nucleotide chain in the sample solution based on the obtained measured values.

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5 The present invention enables the observation of targeted nucleotide chains by fluorescence even under dry conditions. This brings the following good results: the restrictions on the containers used for  
10 detection/quantification of targeted nucleotide chains can be relaxed; even a very slight amount of sample can be used without caring about its getting dried; the detecting/quantifying operations become relatively simpler and easier because of dry conditions; and the direction in which excitation light is illuminated is not restricted.

15 Further, in an aspect in which a hybrid of a targeted nucleotide chain and a probe nucleotide chain is fixed on a substrate at the final stage, nucleotide chains other than the hybrid or the fluorescence dye used can be removed by washing operation, as the need arises. This reduces the background, resulting in higher detection/quantification sensitivity.

20 BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is a graphical representation of detection/quantification of a model targeted nucleotide chain using EB, P2 and YOYO1 by the hybrid nucleotide chain solution method (solution system);

25 FIG. 2 is a graphical representation of detection/quantification of a model targeted nucleotide chain using EB, P2 and YOYO1 by the hybrid nucleotide

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chain solution method (dry method);

FIG. 3 is a graphical representation of detection/quantification of M13mp18 using EB, P2 and YOYO1 by the hybrid nucleotide chain solution method;

5 FIG. 4 is a graphical representation of detection/quantification of mRNA using P2 by the hybrid nucleotide chain solution method;

10 FIG. 5 is a graphical representation of detection/quantification of a model targeted nucleotide chain using EB, P2 and YOYO1 by the probe nucleotide chain fixing method (solution system);

15 FIG. 6 is a graphical representation of detection/quantification of a model targeted nucleotide chain using EB, P2 and YOYO1 by the probe nucleotide chain fixing method (dry method);

FIG. 7 is a graphical representation of detection/quantification of M13mp18 using EB, P2 and YOYO1 by the probe nucleotide chain fixing method;

20 FIG. 8 is a graphical representation of detection/quantification of mRNA using P2 by the probe nucleotide chain fixing method;

25 FIG. 9 is a graphical representation of detection/quantification of a double-stranded nucleotide chain using EB, P2 and YOYO1 (solution system);

FIG. 10 is a graphical representation of detection/quantification of a double-stranded

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#### **nucleotide chain using EB, P2 and YOYO1 (dry method):**

FIG. 11 is a graphical representation of detection/quantification of a triple-stranded nucleotide chain using P2; and

5 FIG. 12 is a graphical representation of  
detection/quantification of a quadruple-stranded  
nucleotide chain using P2.

## DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

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a step (2) of drying the hybrid (C) and the fluorescence dye (D) on the substrate; and  
a step (3) of measuring the fluorescence emitted from the fluorescence dye (D), as a measuring means,  
5 after the drying operation.

In the above step (1), the formation (1-1) of the hybrid (C) and the action (1-2) of the fluorescence dye (D) on the hybrid (C) may be carried out:

10 (a) in state where they are dissolved in the sample solution; or  
(b) in state where they are fixed on the substrate.

The latter (b) case includes a step (1-0) or (1-0') of previously fixing either one of the targeted  
15 nucleotide chain (B) or the probe nucleotide chain (A) on the clean observation solid-phase substrate, and after this step, the formation (1-1) of the hybrid (C) is carried out by allowing the other solution to act on the fixed solid, subsequently the action (1-2) of the  
20 fluorescence dye (D) on the hybrid (C) is carried out.

At this time, as the need arises, the following washing steps may be added:

25 a step (1-1') of removing the nucleotide chain other than the hybrid (C) in the solution by washing operation after the formation (1-1) of the hybrid (C) and before the action (1-2) of the fluorescence dye (D) on the hybrid (C);

a step (1-3) of removing the fluorescence dye (D) not having acted on the hybrid (C) after the action (1-2) of the fluorescence dye (D) on the hybrid (C); and a step (1-4) of removing the solvent including the hybrid (C) and the fluorescence dye (D) having acted on the hybrid (C) by gas flow between the above steps (1) and (2).

In the (b) case, though there are different cases where the probe nucleotide chain (B) is fixed on the solid-phase substrate and the targeted nucleotide chain (A) is fixed on the same, there is basically no difference in means for detecting the hybrid (C) of the targeted nucleotide chains and the probe nucleotide chains finally formed on the solid-phase substrate surface.

According to the present invention, the hybrid (C) of the targeted nucleotide chain and the probe nucleotide chain as well as the fluorescence dye (D) having acted on the above hybrid finally exist on the substrate surface in the form of a dried thin film with a certain area; therefore, handling the sample becomes easier, and the direction of excitation light illumination is not restricted because there exists no liquid. In addition, the restrictions on the sample container used are relaxed substantially, since the fluorescence is measured for the sample in the dried state.

IT GOES WITHOUT SAYING THAT IF THE AMOUNT OF EACH  
SOLUTION USED BEFORE THE STEP (2) IS RELATIVELY LARGE,  
THE CONTAINER IS NEEDED WHICH HAS CONCAVE PORTIONS  
CORRESPONDING TO THE AMOUNT; HOWEVER, IF THE AMOUNT OF  
5 THE SAME IS SUFFICIENTLY SMALL, A PLANAR SUBSTRATE MAY  
BE USED.

HOWEVER, IN THE CASE OF (A), WHEN QUANTITATIVE  
EVALUATION IS REQUIRED, IT BECOMES NECESSARY TO PREPARE  
MICRO CONCAVE PORTIONS WITH WHICH THE AREA OF THE  
10 DROPPED LIQUID CAN BE SPECIFIED.

FURTHER, ACCORDING TO THE PRESENT INVENTION, THE  
STEP IS NOT NECESSARY, IN PRINCIPLE, OF REMOVING THE  
FLUORESCENCE DYE (D) NOT HAVING ACTED ON THE HYBRID BY  
WASHING OPERATION, BECAUSE THE DYE (D) USED FOR ACTING  
15 ON THE TARGETED NUCLEOTIDE CHAINS IN THE SOLUTION IS  
SUCH THAT IT EMITS FLUORESCENCE OR INCREASES ITS  
FLUORESCENCE INTENSITY ONLY AFTER ACTING ON THE HYBRID  
(C) OF THE TARGETED NUCLEOTIDE CHAINS AND THE PROBE  
NUCLEOTIDE CHAINS.

20 HOWEVER, IN THE CASE OF (B), BECAUSE THE HYBRID  
(C) TO BE DETECTED AT THE FINAL STAGE IS FIXED ON THE  
SURFACE OF THE SOLID-PHASE SUBSTRATE, PARTICULARLY WHEN  
USING THE FLUORESCENCE DYE OF A TYPE WHICH INCREASES  
ITS FLUORESCENCE INTENSITY AFTER ACTING ON THE HYBRID,  
25 IT BECOMES EASIER TO REMOVE THE DYE NOT HAVING ACTED ON  
THE HYBRID BY WASHING OPERATION SO AS TO REDUCE THE  
BACKGROUND, AS THE NEED ARISES, AND IT ALSO BECOMES

easier to remove the nucleotide chains other than the hybrid formed on the solid-phase substrate by washing operation so as to reduce the background.

Further, according to the present invention, the  
5 problem of the fluorescence being quenched by drying  
the sample solution can be avoided in principle,  
because the fluorescence dye (D) is used which is  
capable of continuing to emit fluorescence even in the  
dried state while acting on the double-stranded  
10 nucleotide chains and the hybrid.

Any fluorescence dyes may be used in the present  
invention as long as they emit fluorescence or increase  
their fluorescence intensity only after acting on the  
hybrid in a solution and are capable of continuing to  
15 emit fluorescence even in the dried state while acting  
on the hybrid.

The mechanism is not clear for the fluorescence  
dye used in the present invention to act on the  
nucleotide chain hybrid and continue to emit  
20 fluorescence even in the dried state; however,  
considering the fact that in the sole solutions of  
either of FITC and rhodamine, which have high  
fluorescence intensity (from another viewpoint, high  
25 quantum yield of fluorescence) and are relatively  
stable in a solution, their fluorescence is rapidly  
quenched once they are left in the dried state, it is  
presumed that the micro-environment of the fluorescence

dye in the present invention undergoes some change when the dye acts on the nucleotide chain and becomes suitable for the fluorescence emission.

From that viewpoint, the modes in which the 5 fluorescence dye in the present invention acts on the nucleotide chain hybrid are desirably such that its dye molecules are included in the nucleotide chain hybrid.

The fluorescence dye's action modes described above include, for example, intercalation in which the 10 dye molecules enter the space between the two base pairs of the nucleotide chain hybrid, which is a double-stranded nucleotide chain, and groove binding in which the dye molecules enter the groove of the double-stranded nucleotide chain.

15 After the intensive investigation of the fluorescence intensity and safety of the dyes acting on the nucleotide chain hybrid in these modes, the present inventors have found that EB and 2-methyl-4,6-bis(4-N,N-dimethylaminophenyl) pyrylium salt are desirable as 20 intercalators and YOYO1 is desirable as a groove binding type dye. It goes without saying that these dyes are not intended to limit the present invention.

Any nucleotide chains may be the targeted nucleotide chains detectable/quantifiable by the 25 present invention as long as they hybridize with probe nucleotide chains. Both DNA and RNA can be the targeted nucleotide chains of the present invention.

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However, in the case of (a), if there exist in the solution double-stranded nucleotide chains other than the hybrid of the targeted nucleotide chains and the probe nucleotide chains, the double-stranded nucleotide chains other than the hybrid are also detected in principle; thus, the double-stranded nucleotide chains other than the hybrid need to be discriminated and removed. In order to remove such operations, nucleotide chains previously separated into single strands or RNA which is single-stranded from the beginning are desirably used as the targeted nucleotides.

Further, among RNAs, mRNA, of which partially double-stranded portion is small compared with tRNA and rRNA, is more desirably used as the targeted nucleotide chains.

On the other hand, in the case of the method (b), since the nucleotide chains other than the hybrid can be easily removed by washing operation, as described above, both single-stranded and double-stranded nucleotide chains can be subjected to detection as they are by controlling hybridization conditions. In terms of the capability of making the detection easier, single-stranded nucleotide chains may be more desirably used as the targeted nucleotide chains; however, there is basically no difference between the two types nucleotide chains. Among single-stranded nucleotide

chains, mRNA, which has small partially double-stranded portion and codes directly the amino acid sequence, is more desirably used, just like the case of (a).

In the method (b) of the present invention, as the 5 substrate on which a hybrid of a targeted nucleotide chain and a probe nucleotide chain is finally fixed, any substrates may be used as long as the hybrid can be attached thereto. The substrates include, for example, glass substrates formed of synthetic quartz, fused 10 quartz or others and resin substrates formed of acryl, polycarbonate, polystyrene or others.

In the use of the above substrates, according to the method of the present invention, the restrictions are relaxed which are imposed on the excitation light 15 illumination direction when detecting fluorescence; however, when using, for example, a fluorescence microscope which illuminates the substrate, on which the substances to be detected are fixed, with excitation light from its back side, the substrate is 20 required to be optically transparent to the wavelength of both excitation light and fluorescence emitted.

On the other hand, even when using a fluorescence microscope which illuminates the substrate with excitation light from its substance side, in view of 25 the scattering of excitation light, the substrate is desirably optically transparent like the above case or desirably such that it can control the reflection of

excitation light to some extent, like a black mirror finished or black frosted substrate. Translucent substrates are not desirable, especially in view of the scattering of excitation light.

5 As the method of attaching the targeted nucleotide chain or probe nucleotide chain used in the present invention on a substrate, any methods may be adopted as long as they allow the targeted nucleotide chain or probe nucleotide chain to attach on the substrate,  
10 allow the same to form a hybrid with the probe nucleotide chain or targeted nucleotide chain, and allow the hybrid to be detected/quantified by the method of the present invention.

15 In such a case, the nylon filters used in the aforementioned Southern hybridization or the like can also be used in principle; however, in view of the efficiency in hybridization or the aforementioned fluorescence detecting method, desirably a method is adopted to bond targeted nucleotide chain or probe  
20 nucleotide chain covalently on a substrate which is advantageous to optical detection.

25 In recent years, as substrates for use in such a method, polystyrene micro-plates with amino group attached thereon (brand name: Sumiron N) and with carboxyl group attached thereon (brand name: Sumiron C) are commercially available from Sumitomo Bakelite Co., Ltd..

The nucleotide chain can be attached on the substrate by the method which activates the above amino group and the hydroxyl group at 5' terminal of the nucleotide chain with carbonyldiimidazole so as to 5 subject the same to peptide bond, or by the method which subject the above carboxyl group and the nucleotide chain with primary amino group attached thereto to attachment by the method described in published Japanese Laid-Open Application of PCT Patent 10 Application No. 7-506482. Alternatively, the method can also be adopted which introduces a maleimide group to the above carboxyl group, followed by subjecting the carboxyl group and the nucleotide chain with sulphydryl group attached thereto to attachment.

15 In addition, the method can also be adopted which applies an aminosilane coupling agent on a glass substrate, followed by subjecting the amino group and the nucleotide chain to attachment in the same manner as described above, or which applies a glycidylsilane 20 coupling agent on a glass substrate, followed by subjecting the epoxy group and the amino group attached to the nucleotide chain or amino group of the nucleotide chain base to attachment in such a manner as not to interfere with hybridization.

25 The substrate have only to be in the dried state which enables the elimination of the obstacles to fluorescence measurements due to the existence of water

having a flow property or due to its containing moisture. The dry conditions under which water does not interfere with fluorescence measurements should be determined, for example, based on the data obtained by 5 preparing a sample equivalent to that of the actual measurements, conducting a pretest in accordance with the same sample drying procedure as an actual one, and making NMR measurements for the dried sample.

The concrete drying methods include, for example, 10 the drying-up method using a vacuum pump as adopted in the following examples; however, the methods are not limited to such an example and any methods can be adopted as long as they have the effect equivalent to that of the drying-up method.

15 The detection/quantification of multi-stranded nucleotide chains of the present invention are characterized in that they comprise the steps of:

(1) adding to a sample solution, which is subjected to detection/quantification of multi-stranded 20 nucleotide chains, a fluorescence dye having a fluorescence characteristic of emitting fluorescence or increasing its fluorescence intensity in the presence of the multi-stranded nucleotide chains and capable of maintaining the fluorescence characteristic even in the 25 dried state;

(2) placing a known amount of the sample solution with the fluorescence dye added thereto on a clean

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observation substrate so as to dry the same; and

(3) measuring the fluorescence emitted from the dried sample and detecting/quantifying the multi-stranded nucleotide chains in the sample solution based 5 on the obtained measured values.

According to the present invention, multi-stranded nucleotide chains as well as the fluorescence dye having acted on the above multi-stranded nucleotide chains exist on the substrate surface in the form of a 10 dried thin film with a certain area at the final stage; therefore, handling the sample becomes easier, and the direction of excitation light illumination is not restricted. In addition, the restrictions on the sample container used are relaxed substantially, since 15 the fluorescence is measured for the sample in the dried state.

It goes without saying that if the amount of the solution dropped in the step (2) is relatively large, the container is needed which has concave portions 20 corresponding to the amount; however, if the amount of the same is sufficiently small, a planar substrate may be used. However, when quantitative evaluation is required, it becomes necessary to prepare micro concave portions with which the area of the dropped liquid can 25 be specified.

Further, according to the present invention, the step is not necessary, in principle, of removing the

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fluorescence dye not having acted on the multi-stranded nucleotide chains by washing operation, because the dye used for acting on the multi-stranded nucleotide chains in the solution is such that it emits fluorescence or 5 increases its fluorescence intensity only after acting on the multi-stranded nucleotide chains. In addition, the problem of the fluorescence's being quenched by drying the sample solution, as described above, can be avoided in principle, because the fluorescence dye is 10 used which is capable of continuing to emit fluorescence even in the dried state while acting on the multi-stranded nucleotide chains.

Any fluorescence dye may be used in the present invention as long as it emits fluorescence or increases 15 its fluorescence intensity only after acting on the multi-stranded nucleotide chains in the solution and is capable of continuing to emit fluorescence even in the dried state while acting on the multi-stranded nucleotide chains.

20 The mechanism is not clear for the fluorescence dye used in the present invention to act on the multi-stranded nucleotide chains and continue to emit fluorescence even in the dried state; however, considering the fact that in the sole solutions of 25 either of FITC and rhodamine, which have high fluorescence intensity (from another viewpoint, high quantum yield of fluorescence) and are relatively

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stable in a solution, their fluorescence is rapidly quenched once they are left in the dried state, it is presumed that the micro-environment of the fluorescence dye in the present invention undergoes some change when 5 the dye acts on the multi-stranded nucleotide chain and becomes suitable for the fluorescence emission.

From that viewpoint, the modes in which the fluorescence dye in the present invention acts on the multi-stranded nucleotide chain are desirably such that 10 its dye molecules are included in the multi-stranded nucleotide chain. The fluorescence dye's action modes described above include, for example, intercalation in which the dye molecules enter the space between the two base pairs of the multi-stranded nucleotide chains 15 including double-stranded nucleotide chains, and groove binding in which the dye molecules enter the groove of the multi-stranded nucleotide chain.

After the intensive investigation of the fluorescence intensity and safety of the dyes acting on 20 the multi-stranded nucleotide chains in these modes, the present inventors have found that EB and 2-methyl-4,6-bis(4-N,N-dimethylaminophenyl) pyrylium salt are desirably used as intercalators and YOYO1 is desirably used as a groove binding type dye in the dry 25 detection/quantification of multi-stranded nucleotide chains by fluorescence.

It goes without saying that these dyes are not

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intended to limit the present invention.

The multi-stranded nucleotide chains detectable/quantifiable by the present invention may include not only the ordinary double-stranded DNA, but 5 also the triple-stranded DNA and quadruple-stranded DNA as described above, in addition, natural type multi-stranded nucleotide chains such as DNA-RNA hybrid and RNA-RNA hybrid, multi-stranded nucleotide chains of which backbone has a non-natural type structure, such 10 as those at least one strand of which is phosphorothionate-type or hydrogenphosphonate-type or protein nucleotide chains of which backbone consists of nucleotide, and non-natural type nucleotide chains of which saccharified portion or base portion is modified. 15 And any nucleotide chains may be included as long as they are capable of forming multi-stranded chains by recognizing their partners with hydrogen bond between bases.

The substrate have only to be in the dried state  
20 which enables the elimination of the obstacles to  
fluorescence measurements due to the existence of water  
having a flow property or due to its containing  
moisture. The dry conditions under which water does  
not interfere with fluorescence measurements should be  
25 determined, for example, based on the data obtained by  
preparing a sample equivalent to that of the actual  
measurements, conducting a pretest in accordance with

the same sample drying procedure as an actual one, and making NMR measurements for the dried sample.

The concrete drying methods include, for example, the drying-up method using a vacuum pump as adopted in the following examples; however, the methods are not limited to such an example and any methods can be adopted as long as they have the effect equivalent to that of the drying-up method.

In the following, the present invention will be described in detail with reference to the following examples.

(Example 1)

Detection of Oligodeoxynucleotide with EB, 2-Methyl-4,6-bis(4-N,N-Dimethylaminophenyl) Pyrylium Iodide (hereinafter referred to as P2) and YOYO1

(1) 20-mer oligodeoxynucleotide having completely the same base sequence as the specific part of that of M13mp18, which is a single-stranded DNA, was obtained (from Kanto Chemical Co., Ltd.) as a model targeted nucleotide chain. The base sequence was as follows:

5 'ACTGGCCGTCGTTTACAAC3'

(SEQ ID NO: 1)

A 100 µM, in terms of the base concentration, stock solution of the model targeted nucleotide chain was prepared by properly mixing the aqueous solution of the above oligonucleotide and water.

(2) 20-mer oligodeoxynucleotide having base

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sequence complementary to that of the above model targeted nucleotide chain was obtained as a probe nucleotide chain, and a 100 pM, in terms of the base concentration, stock solution of the probe nucleotide chain was prepared in the same manner as described in (1). The base sequence was as follows:

3' TGACCGGGCAGCAAAATGTTG5'

(SEQ ID NO: 2)

(3) Four mg of EB (from Sigma Ardrich Japan) was dissolved in 1 mL of DMSO and 9 mL of water was added. Additional water was added to the solution to bring about a 100-fold dilution as a 10  $\mu$ M stock solution.

(4) Five mg of P2 (synthesized by the present inventors) was dissolved in 1 mL of acetonitrile and 9 mL of water was added. Additional water was added to the solution to bring about a 100-fold dilution as a 10  $\mu$ M stock solution.

(5) Water was added to YOYO1 (from Molecular Probe Co., 1mM/DMSO) to bring about a 100-fold dilution as a 10  $\mu$ M stock solution.

(6) Each of 0, 2, 5, 10  $\mu$ L of model targeted nucleotide chain solutions (final concentrations of 0, 2.0, 5.0 and 10.0  $\mu$ M, respectively, at the stage where a dye solution has been added), 15  $\mu$ L of probe nucleotide chain solution (a final concentration of 15  $\mu$ M) and 5  $\mu$ L of 100 mM Tris-HCl buffer solution (pH 7.5) were mixed and water was added to a volume of 90

µL. Each solution was annealed and cooled to a final temperature of 4°C.

(7) Ten  $\mu$ L (final concentration 1  $\mu$ M) of each dye solution was added to each of the above DNA solutions.

5 (8) 0.5  $\mu$ L of each solution (5) was placed on a  
properly cleaned and dried transparent acrylic  
substrate 1 mm thick (from Asahi Chemical Industry Co.,  
Ltd., Deluglass A). For the solutions in the above  
state and another solutions having been subjected to  
0 the same operation as above and the drying-up by a  
vacuum pump, the fluorescence was observed and the  
intensity of the same was measured.

For the fluorescence observation, used was an inverted-type fluorescence microscope IMT2 (objective lens of 10 magnifications) from Olympus Optical Co., Ltd. And as the filter cubes for the fluorescence observation, a G excitation filter was used for each case using EB, a B excitation filter for each case using YOYO1 and a filter specially prepared (by Asahi Bunko, excitation: 580 nm, fluorescence: 540 nm, dichroic mirror: 610 nm) for each case using P2.

For the measurements of fluorescence intensity, used were a CCD equipped with an image intensifier (from Hamamatsu Photonics, ICCD C2400-87) and an image processing apparatus (from Hamamatsu Photonics, Argus 50) which were connected to the above fluorescence microscope via a relay lens (NFK 2.5 × LD).

The measurements of fluorescence intensity were carried out in the area of  $200 \times 200 \mu\text{m}$  which was considered to have the average brightness and be uniform to some extent, and the average values on the pixels ( $2 \times 2 \mu\text{m}$ ) in the area were adopted as measured values.

The amplification degree of the image intensifier was 0.2 in terms of the indicated value. The fluorescence intensity values of the dyes cannot be directly compared because the spectral characteristics of the filters are different from each other, the excitation light source (high pressure mercury lamp) consists of bright lines, and because the sensitivity of the ICCD camera depends on wavelength.

15                   The measured values of the fluorescence intensity  
are shown in FIGS. 1 (solution system) and 2 (dry  
type). It is apparent from FIGS. 1 and 2 that the  
detection/quantification of model targeted nucleotide  
chains can be performed even in the dry-up state with  
any one of EB, P2 and YOYO1, though there are observed  
differences in fluorescence intensity difference and  
linearity. The comparison of the three types of dyes  
shows that the degree of fluorescence quenching is high  
in EB and YOYO1, especially in EB, in the dried up  
state. On the other hand, the degree of fluorescence  
quenching is relatively low in P2 in the dried up  
state; accordingly, P2 can be said to be suitable for

the measuring method of the present invention.

(Example 2)

Detection of Targeted DNA with EB, P2 and YOYO1

(1) M13mp18 (from Takara Shuzo Co., Ltd.), which  
5 is a single-stranded DNA, was obtained as a targeted  
nucleotide chain. The targeted base sequence was the  
same as in Example 1, as shown below:

5'ACTGGCCGTCGTTTACAAC3'

(SEQ ID NO: 1)

10 A 10  $\mu$ M, in terms of the base concentration of the  
targeted portion, a stock solution of the targeted  
nucleotide chain was prepared by properly mixing the  
aqueous solution of the above DNA and water. The  
reason that the concentration of the base of the  
15 targeted portion was dilute compared with that of  
Example 1 is that, since M13mp18 consists of 7249  
bases, the concentration as a nucleotide chain had to  
be kept properly.

(2) Ten  $\mu$ M aqueous solution of  
20 oligodeoxynucleotide having the same base sequence as  
in Example 1, as shown below, was prepared as a probe  
nucleotide chain stock solution.

3'TGACCGGCAGCAAAATGTTG5'

(SEQ ID NO: 2)

25 (3) Ten  $\mu$ M stock solutions of EB, P2 and YOYO1  
were prepared in the same manner as in Example 1.

(4) Each of 0, 2, 5, 10  $\mu$ L of targeted nucleotide

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chain solutions (final concentrations of 0, 0.2, 0.5 and 1.0  $\mu$ M, respectively, at the stage where a dye solution has been added), 15  $\mu$ L of probe nucleotide chain solution (a final concentration of 1.5  $\mu$ M) and 5 5  $\mu$ L of 100 mM Tris-HCl buffer solution (pH 7.5) were mixed and water was added to a volume of 99  $\mu$ L. Each solution was annealed and cooled to a final temperature of 4°C.

10 (5) 1.0  $\mu$ L (final concentration 0.1  $\mu$ M) of each dye solution was added to each of the above DNA solutions.

15 (6) The fluorescence intensity of the sample was measured in the same manner as in Example 1 in state where the sample was dried up.

20 The amplification degree of the image intensifier was 1.0 in terms of the indicated value, because the concentration of the targeted DNA was low.

The measured results are shown in FIG. 3. It is apparent from FIG. 3 that the targeted single-stranded 20 nucleotide chains can be detected/quantified by the method of the present invention.

(Example 3)

#### Detection of Targeted mRNA with P2

142 (1) Human  $\beta$ 2-adrenergic receptor mRNA was synthesized from human  $\beta$ 2-adrenergic receptor cDNA using T7RNA polymerase by conventional procedure and purified after the D Nase treatment. A 10  $\mu$ M, in terms

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25

of the base concentration of the targeted portion, a stock solution of a targeted nucleotide chain was prepared by properly mixing the aqueous solution of the above mRNA and water.

5 The portion of the targeted base sequence of the  
above mRNA was as follows:

5' TGCTGTGCGTCACGGCCAGCAT3'

(SEQ ID NO: 3)

(2) Ten  $\mu\text{M}$  aqueous solution of

10 oligodeoxynucleotide (from Kanto Chemical Co., Ltd.)  
having the following base sequence was prepared as a  
probe nucleotide chain stock solution.

3' ACGACACGCAGTGCCGGTCGTA5'

(SEQ ID NO: 4)

15 (3) Ten  $\mu$ M stock solution of P2 was prepared in  
the same manner as in Example 1.

(4) Each of 0, 2, 5, 10  $\mu$ L of targeted nucleotide chain solutions (final concentrations of 0, 0.2, 0.5 and 1.0  $\mu$ M, respectively, at the stage where a dye solution has been added), 15  $\mu$ L of probe nucleotide chain solution (a final concentration of 1.5  $\mu$ M) and 5  $\mu$ L of 100 mM Tris-HCl buffer solution (pH 7.5) were mixed and water was added to a volume of 90  $\mu$ L. And the solution was annealed and cooled to a final temperature of 4°C.

(5) 1.0  $\mu$ L (final concentration 0.1  $\mu$ M) of P2 solution was added to each of the above DNA solutions.

(6) The fluorescence intensity of the sample was measured in the same manner as in Example 2 in state where the sample was dried up.

5 The measured results are shown in FIG. 4. It is apparent from FIG. 4 that the targeted mRNA can be detected/quantified by the method of the present invention.

(Example 4)

10 Detection of Oligodeoxynucleotide with EB, 2-methyl-4,6-bis(4-N,N-dimethylaminophenyl) pyrylium iodide (hereinafter referred to as P2) and YOYO1 (probe nucleotide chain being fixed)

15 (1) The same 20-mer oligodeoxynucleotide (from Kanto Chemical Co., Ltd.) as used in Example 1 was used as a model targeted nucleotide chain. The base sequence was as follows:

5 'ACTGGCCGTCGTTTACAAC3'

(SEQ ID NO: 1)

20 A 100  $\mu$ M, in terms of the base concentration, stock solution of the model targeted nucleotide chain was prepared by properly mixing the aqueous solution of the above oligonucleotide and water.

25 *IASA3* (2) 20-mer oligodeoxynucleotide having base sequence complementary to that of the above model targeted nucleotide chain was obtained as a probe nucleotide chain, and a 100  $\mu$ M, in terms of the base concentration, stock solution of the probe nucleotide

a chain was prepared in the same manner as described in  
(1). In order to fix the probe nucleotide chain on a  
solid-phase substrate by the covalent bond, 20-mer  
oligodeoxynucleotide was obtained and used at which 5'  
5 terminal an amino group was attached using  
hexamethylene as a linker. The base sequence was as  
follows:

*a* 3' TGACCGGCAGCAAAATGTTG-NH25'

(SEQ-ID-NO: 2)

10 (3) Ten  $\mu$ M stock solutions of EB, P2 and YOYO1  
were prepared in the same manner as in Example 1.

(4) The probe nucleotide chain of (2) was fixed on a 96-well micro-plate with carboxyl groups attached to its surface (from Sumitomo Bakelite Co., Ltd.,

15 Sumiron C) using 1-hydroxy-2,5-dioxo-3-  
pyrrolidinesulfonic acid monosodium salt (from Sigma  
Ardrich Japan, hereinafter referred to as sulfoNHS) and  
1-ethyl-3-(3 dimethylaminopropyl)carbodiimide  
hydrochloride (from Sigma Ardrich Japan, hereinafter  
20 referred to as EDC) in accordance with the method  
disclosed in the published Japanese Laid-Open  
Application of PCT Patent Application.

(5) Each of 0, 2, 5, 10  $\mu$ L of the model targeted nucleotide chain solutions (final concentrations of 0, 2.0, 5.0 and 10.0  $\mu$ M, respectively) and 5  $\mu$ L of 100 mM Tris-HCl buffer solution (pH 7.5) were mixed in the wells of the above micro-plate and water was added to

100  $\mu$ L. And the solution were annealed and cooled to a final temperature of 4°C. Each of the following steps before fluorescence observation was carried out at 4°C.

5 (6) Since it was considered that the targeted nucleotide chain was used in excess of the probe nucleotide chain attached on the well surface of the micro-plate, the wells were emptied and washed with 100  $\mu$ L of the above buffer solution three times, and 90  $\mu$ L of the above buffer solution was added.

10 (7) Ten  $\mu$ L (final concentration of 1  $\mu$ M) of each dye solution was added to the above DNA solution and left stand for about 10 minutes, then each dye solution was removed by suction.

15 (8) The wells were washed with 100  $\mu$ L of the above buffer solution once, and this solution was removed by suction.

20 (9) The micro-plate was dried up with a vacuum pump, and the fluorescence was observed and the intensity of the same was measured. In addition to this, fluorescence was measured for the solution system to which 100  $\mu$ L of 10 mM Tris-HCl buffer solution (pH 7.5) was added after the step (10).

The amplification degree of the image intensifier was 4.0 in terms of the indicated value.

25 The measured values of the fluorescence intensity are shown in FIGS. 5 (solution system) and 6 (dry type). It is apparent from FIGS. 5 and 6 that the

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detection/quantification of model targeted nucleotide  
chains can be performed even in the dry-up state with  
any one of EB, P2 and YOYO1, though there are observed  
differences in fluorescence intensity difference and  
linearity. The comparison of the three types of dyes  
shows that the degree of fluorescence quenching is high  
in EB and YOYO1, especially in EB, in the dried up  
state. On the other hand, the degree of fluorescence  
quenching is relatively low in P2 in the dried up  
state; accordingly, P2 can be said to be suitable for  
the measuring method of the present invention.

(Example 5)

Detection of Targeted DNA with P2 (probe  
nucleotide chain being fixed)

(1) M13mp18 (from Takara Shuzo Co., Ltd.), the  
same single-stranded DNA as used in Example 2, was used  
as a targeted nucleotide chain. The targeted base  
sequence was the same as in Examples 1 and 4, as shown  
below:

5 'ACTGGCCGTCGTTTACAAC3'

(SEQ ID NO: 1)

A 10  $\mu$ M, in terms of the base concentration of the  
targeted portion, stock solution of the targeted  
nucleotide chain was prepared by properly mixing the  
aqueous solution of the above DNA and water. The  
reason that the concentration of the base of the  
targeted portion was dilute compared with that of

Example 5 is that, since M13mp18 consists of 7249 bases, the concentration as a nucleotide chain had to be kept properly.

*INS A4* 5 (2) 20-mer oligodeoxynucleotide having base sequence complementary to that of the above model targeted nucleotide chain was obtained as a probe nucleotide chain in the same manner as in Example 4, and a 100 µM, in terms of the base concentration, stock solution of the probe nucleotide chain was prepared in 10 the same manner as described in (1). The base sequence was as follows:

3' TGACCGGGAGCAAAATGTTG-NH25'

(SEQ ID NO: 2)

15 (3) The probe nucleotide chain of (2) was fixed on a micro-plate in the same manner as in Example 4.

(4) Each of 0, 2, 5, 10 µL of the targeted nucleotide chain solutions (1) (final concentrations of 0, 0.2, 0.5 and 1.0 µM, respectively) and 5 µL of 100 mM Tris-HCl buffer solution (pH 7.5) were mixed in the 20 wells of the above micro-plate and water was added to 100 µL. And the solutions were annealed and cooled to a final temperature of 4°C. Each of the following steps before fluorescence observation was carried out at 4°C.

25 (5) The wells were emptied and washed with 100 µL of the above buffer solution three times, and 90 µL of the above buffer solution was added.

(6) Ten  $\mu$ L (final concentration of 1  $\mu$ M) of P2 solution prepared in the same manner as in Example 4 was added to the above DNA solution and left stand for about 10 minutes, then the dye solution was removed by 5 suction.

(7) The wells were washed with 100  $\mu$ L of the above buffer solution once, and this solution was removed by suction.

10 (8) The fluorescence intensity was measured in state the sample was dried up in the same manner as in Example 4.

15 The measured values of the fluorescence intensity are shown in FIG. 7. It is apparent from FIG. 7 that the targeted single-stranded DNA can be detected/quantified by the method of the present invention.

(Example 6)

Detection of Targeted mRNA with P2 (probe nucleotide chain being fixed)

20 ~~145A5~~ (1) Human  $\beta$ 2 adrenergic receptor mRNA was synthesized from human  $\beta$ 2 adrenergic receptor cDNA using T7RNA polymerase by conventional procedure and purified after the D Nase treatment. A 10  $\mu$ M stock solution of targeted nucleotide chain was prepared as a  
25 base of the targeted portion by properly mixing the aqueous solution of the above mRNA and water.

The portion of the targeted base sequence of the

above mRNA was as follows:

5 'TGCTGTGCGTCACGGCCAGCAT3'

(SEQ ID NO: 3)

*INSR* 5 (2) 146 Ten  $\mu$ M aqueous solution of oligodeoxynucleotide (from Kanto Chemical Co., Ltd.) having the following base sequence was prepared as a probe nucleotide chain stock solution. An amino group was attached at the 5' terminal of this oligodeoxynucleotide like Examples 4 and 5.

10 3 'ACGACACCCAGTGGCGGTGCTA-NH25'

(SEQ ID NO: 4)

(3) The probe nucleotide chain of (2) was fixed on a micro-plate in the same manner as in Example 4.

15 (4) Each of 0, 2, 5, 10  $\mu$ L of targeted mRNA solutions (final concentrations of 0, 0.2, 0.5 and 1.0  $\mu$ M, respectively) and 5  $\mu$ L of 100 mM Tris-HCl buffer solution (pH 7.5) were mixed in the wells of the above micro-plate and water was added to a volume of 100  $\mu$ L. And the solution was annealed and cooled to a final 20 temperature of 4°C. Each of the following steps before fluorescence observation was carried out at 4°C.

(5) The wells were emptied and washed with 100  $\mu$ L of the above buffer solution three times, and 90  $\mu$ L of the above buffer solution was added.

25 (6) Ten  $\mu$ L (final concentration of 1  $\mu$ M) of P2 solution prepared in the same manner as in Example 4 was added to the above solution and left stand for

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about 10 minutes, then the dye solution was removed by suction.

(7) The wells were washed with 100  $\mu$ L of the above buffer solution once, and this solution was 5 removed by suction.

(8) The fluorescence intensity was measured in state the sample was dried up in the same manner as in Example 4.

10 The measured results are shown in FIG. 8. It is apparent from FIG. 8 that the targeted mRNA can be detected/quantified by the method of the present invention.

(Example 7)

15 Detection of Targeted DNA with P2 (targeted nucleotide chain being fixed)

(1) pUC18, which is double-stranded DNA, was obtained (from Takara Shuzo Co., Ltd.) as a targeted nucleotide chain. The targeted base sequence was the same as in Examples 5 and 6, which was on the outside 20 of the restriction enzyme (Hind III) digestion sites at a multiple cloning site. The base sequence was as follows:

5'ACTGGCCGTCGTTTACAAC3'

(SEQ ID NO: 1)

25 (2) The above DNA was subjected to complete restriction digestion with EcoR I (from Takara Shuzo Co., Ltd.) by a certain method and recovered by ethanol

precipitation.

(3) The recovered DNA was further subjected to complete restriction digestion with Pvu II (from Takara Shuzo Co., Ltd.) and recovered by ethanol 5 precipitation. The recovered DNAs after the restriction enzyme digests contained the following three types of DNAs:

- 1) double-stranded DNA with blunt ends on both ends of about 2000 base pairs;
- 10 2) double-stranded DNA with a blunt end on one end of about 100 base pairs and a sticky end 3' TTAA (AA being on the terminal side) on the other end of the same; and
- 15 3) double-stranded DNA with a blunt end on one end of about 200 base pairs and a sticky end 3' TTAA (AA being on the terminal side) on the other end of the same.

The targeted base sequence of (1) exists on the sense strand side having the sticky end.

20 (4) Two % solution (water : ethanol = 1 : 1) of silane coupling agent with epoxy group (from Shin-Etsu Chemical Co., Ltd., KBM 403,  $\gamma$ -glycidoxypropyltrimethoxysilane) was spin coated on a properly cleaned quartz glass substrate (from Iiyama Special Glass, 25.4 x 25.4 mm, 0.5 mm thick) and dried 25 at 100°C for 1 hour. Thus epoxy group was attached on the substrate.

(5) The glass substrate of (4) was reacted with 1  $\mu$ M DNA solution of (3) (10 mM phosphoric acid buffer solution, pH 7.0, 2 mL) at room temperature for 24 hours. As a result, the amino group of adenine at the 5 pairing end of the double-stranded DNAs of (3)-2) and -3) was reacted with the epoxy group on the substrate and attached thereto.

(6) The above glass substrate was water-washed, heated in 2 mL of water at 95°C for 10 minutes, and 10 washed with hot water at 95°C. As a result, of the double-stranded DNAs of (3)-2) and -3), each single strand with its pairing end attached on the substrate alone remained on the substrate.

(7) The above substrate was immersed in 2 mL of 1  $\mu$ M, in terms of the base concentration of the probe 15 nucleotide chain having the same base sequence as those of Examples 4 and 5 (obtained from Kanto Chemical Co., Ltd., with no amino group attached), solution (100 mM Tris-HCl, pH 7.5) so as to be under hybridization 20 conditions and cooled to a final temperature of 4°C. Thus, the probe nucleotide chain formed a hybrid with the DNA attached on the substrate. Each of the following operations before fluorescence detection was carried out at 4°C.

25 (8) The above glass substrate was washed with 2 mL of the buffer solution of (7) three times and immersed in 2 mL of P2 solution (1  $\mu$ M) prepared in the

same manner as in Example 4 for 10 minutes.

(9) The substrate was washed with 2 mL of the above buffer solution, and the liquid on the substrate was removed with argon gas flow.

5 (10) The fluorescence intensity was measured in  
state where the sample was dried up, in the same manner  
as in Example 4. As a control, measurements were made  
for the glass substrate which had not been subjected to  
the attachment operation of (6). The measured results  
0 are shown in Table 1.

Table 1 pUC18 Detection Result

	Sample	Blank
Fluorescence Intensity	116	2

It is apparent from Table 1 that the targeted nucleotide chains attached on the substrate can be detected by the method of the present invention.

(Comparative Example 1)

## Observation of FITC Fluorescence under Dry Conditions

One  $\mu\text{M}$  aqueous solution of FITC (from Molecular Probe Co.), to which amino group had been introduced taking into consideration its solubility in water, was prepared, and a proper amount of the same was allowed to penetrate between a slide glass and a cover glass. The fluorescence was observed with a system of a fluorescence microscope (G excitation filter)+ ICCD +

Argus 50, as in the above example.

As a result, relatively intense fluorescence was observed while water existed between the two glasses, however, no fluorescence was observed in the area where 5 water had been evaporated.

(Example 8)

Detection of Double-stranded Nucleotide Chain with  
EB, 2-methyl-4,6-bis(4-N,N-dimethylaminophenyl)  
pyrylium iodide (hereinafter referred to as P2) and  
10 YOYO1

(1) One mg of salmon testes DNA (double-stranded DNA, from Sigma Ardrich Japan) was dissolved in 1 mL of 10 mM Tris-HCl buffer solution (pH 7.5) and subjected to ultrasonic breaking properly, so as to obtain DNA 15 with the average length of 200 to 300 base pairs. The DNA length was confirmed by the agarose electrophoresis. This solution was properly diluted with water so as to obtain a 100  $\mu$ M stock solution in terms of base pair.

20 (2) Four mg of EB (from Sigma Ardrich Japan) was dissolved in 1 mL of DMSO, and 9 mL of water was added thereto. Additional water was added to the solution to bring about a 100-fold dilution as a 10  $\mu$ M stock solution.

25 (3) Five mg of P2 (synthesized by the present inventors) was dissolved in 1 mL of acetonitrile and 9 mL of water was added. Additional water was added to

the solution to bring about a 100-fold dilution as a 10  $\mu\text{M}$  stock solution.

(4) Water was added to YOYO1 (from Molecular Probe Co., 1mM/DMSO) to bring about a 100-fold dilution 5 as a 10  $\mu\text{M}$  stock solution.

(5) Each of 10  $\mu\text{L}$  of dye solutions (final concentration of 1  $\mu\text{M}$ ) and each of 0, 2, 5, 10  $\mu\text{L}$  of DNA solutions (final concentrations of 0, 2.0, 5.0 and 10.0  $\mu\text{M}$ , respectively) were mixed, and water was added to 10 100  $\mu\text{L}$ . Since YOYO1 is unstable when the concentration of salt is low, 10  $\mu\text{L}$  of 100 mM Tms-HCl buffer solution (pH 7.5) were added to a total volume of 100  $\mu\text{L}$ .

(6) 0.5  $\mu\text{L}$  of each solution (5) was placed on a properly cleaned and dried transparent acrylic 15 substrate 1 mm thick (from Asahi Chemical Industry Co., Ltd., Deluglass A). For the solutions in the above state and another solutions having been subjected to the same operation as above and to the drying-up by a vacuum pump, the fluorescence was observed and the 20 intensity of the same was measured. The measurements of fluorescence were promptly carried out in a short time, since the sample might evaporate.

For the fluorescence observation, used was an inverted-type fluorescence microscope IMT2 (objective 25 lens of 10 magnifications) from Olympus Optical Co., Ltd. And as the filter cubes for the fluorescence observation, a G excitation filter was used for each

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case using EB, a B excitation filter for each case using YOYO1 and a filter specially prepared (by Asahi Bunko, excitation: 580 nm, fluorescence: 540 nm, dichroic mirror: 610 nm) for each case using P2.

5 For the measurements of fluorescence intensity, used were a CCD equipped with an image intensifier (from Hamamatsu Photonics, ICCD C2400-87) and an image processing apparatus (from Hamamatsu Photonics, Argus 50) which were connected to the above fluorescence 10 microscope via a relay lens (NFK 2.5 × LD).

The measurements of fluorescence intensity were carried out in the area of  $200 \times 200 \mu\text{m}$  which was considered to have the average brightness and be uniform to some extent, and the average values on the 15 pixels ( $2 \times 2 \mu\text{m}$ ) in the area were adopted as measured values. The amplification degree of the image intensifier was 0.2 in terms of the indicated value.

The fluorescence intensity values of the dyes cannot be directly compared because the spectral 20 characteristics of the filters are different from each other, the excitation light source (high pressure mercury lamp) consists of bright lines, and because the sensitivity of the ICCD camera depends on wavelength.

The measured values of the fluorescence intensity 25 are shown in FIGS. 9 (solution system) and 10 (dry type). It is apparent from FIGS. 9 and 10 that the detection/quantification of double-stranded DNA can be

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performed even in the dry-up state with any one of EB, P2 and YOYO1, though there are observed differences in fluorescence intensity difference and linearity.

The comparison of the three types of dyes shows  
5 that the degree of fluorescence quenching is high in EB and YOYO1, especially in EB, in the dried up state. On the other hand, the degree of fluorescence quenching is relatively low in P2 in the dried up state; accordingly, P2 can be said to be suitable for the  
10 measuring method of the present invention.

(Example 9)

Quantification of Triple-stranded Nucleotide Chain  
with P2

(1) The following 3 types of synthesized 11-mer  
15 oligodeoxynucleotides, which form parallel type of triple-stranded chains, were purchased (from Kanto Chemical Co., Ltd.).

a) 5' TTCTTCTTTTC3'

(SEQ ID NO: 5)

20 b) 3' AAGAAGAAAAG5'

(SEQ ID NO: 6)

c) 3' TTCTTCTTTTC5'

(SEQ ID NO: 7)

Each of 500  $\mu$ M solutions, in terms of the base  
25 pair concentration, of the above oligodeoxynucleotides was annealed in 10 mM phosphoric acid buffer solution (pH 6.5) containing 1M NaCl and 10 mM EDTA (cooled to a

final temperature of 4°C) so as to form a triple-stranded chain and to prepare a 500  $\mu$ M, in terms of the base trio concentration, (1/3 of the entire base concentration) stock solution. The conditions, such as 5 salt concentration, adopted were considered to be suitable for the formation of triple-stranded chains.

(2) Ten  $\mu$ L of P2 solution used in Example 8 and each of 0, 0.5, 1.0 and 2.0  $\mu$ L (final concentrations of 10 0, 2.5, 5.0 and 10.0  $\mu$ M) of DNA solutions of (1) were mixed, and water was added to each solution to a final volume of 100  $\mu$ L.

(3) The fluorescence intensity was measured in state where the sample was dried up, in the same manner as in Example 8.

15 The measured results are shown in FIG. 11. In order to make possible the comparison with the results of Example 1, the amplification degree of the image intensifier and the ordinate of the graph were the same as those of Example 1. It is apparent from FIG. 11 20 that triple-stranded nucleotide chains can be detected/quantified by the method of the present invention.

(Example 10)

Quantification of Quadruple-stranded Nucleotide  
25 Chain with P2

*INS A7* *La7* (1) The following synthesized oligodeoxynucleotide, which has human telomere sequence

*A*  
contributing to the formation of quadruple-stranded chains, were purchased (from Kanto Chemical Co., Ltd.).

d(TTGGG)<sup>2</sup>

400  $\mu$ M solution of the above oligodeoxynucleotide  
5 was annealed in 20 mM Tris-HCl buffer solution (pH 7.2)  
containing 10 mM K<sup>+</sup> (cooled to a final temperature of  
4°C) so as to form a quadruple-stranded chain and to  
prepare a 100  $\mu$ M, in terms of the base quartet  
concentration, (1/4 of the entire base concentration)  
10 stock solution.

(2) Ten  $\mu$ L of P2 solution used in Example 8 and  
each of 0, 2, 5 and 10  $\mu$ L (final concentrations of 0,  
2.0, 5.0 and 10.0  $\mu$ M) of DNA solutions of (1) were  
mixed, and water was added to each solution to a final  
15 volume of 100  $\mu$ L.

(3) The fluorescence intensity was measured in  
state where the sample was dried up, in the same manner  
as in Example 9.

The measured results are shown in FIG. 12. In  
20 order to make possible the comparison with the results  
of Examples 8 and 9, the amplification degree of the  
image intensifier and the ordinate of the graph were  
the same as those of Examples 8 and 9. It is apparent  
from FIG. 12 that quadruple-stranded nucleotide chains  
25 can be detected/quantified by the method of the present  
invention.

(Comparative Example 2)

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Observation of FITC Fluorescence under Dry  
Conditions

One  $\mu$ M aqueous solution of FITC (from Molecular Probe Co.), to which amino group had been introduced 5 taking into consideration its solubility in water, was prepared, and a proper amount of the same was allowed to penetrate between a slide glass and a cover glass. The fluorescence was observed with a system of a 10 fluorescence microscope (G excitation filter) + ICCD + Argus 50, as in the above examples.

As a result, relatively intense fluorescence was observed while water existed between the two glasses, however, no fluorescence was observed in the area where water had been evaporated.